Distribution, Metabolism and Tumoricidal Activity of Doxorubicin Administered in Sorbitan Monostearate (Span 60) Niosomes in the Mouse

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Purpose. Encapsulation of doxorubicin in niosomes was sought as a route to tumour targeting and improved tumoricidal through the alteration of doxorubicin pharmacokinetics and metabolism. ***Methods.*** Doxorubicin niosomes (10 mg kg^{-1} doxorubicin) prepared from sorbitan monostearate (Span 60), cholesterol and choleth-24 (a 24 oxyethylene cholesteryl ether) in the molar ratio 45:45:10 were administered intravenously to female NMRI mice bearing the MAC 15A subcutaneously implanted tumour. Plasma doxorubicin was fractionated by gel filtration and quantified by HPLC with fluorometric detection as niosome-associated doxorubicin and released doxorubicin. Tumoricidal activity of the formulation was assessed by the intravenous injection of 5 mg kg^{-1} and 10 mg kg^{-1} doxorubicin niosomes to male NMRI mice bearing a 6 day old MAC 15A tumour. ***Results.*** At least 90% of the plasma doxorubicin was associated with the niosome fraction 4 h after dosing, and 50% was still associated after 24 h. The clearance of doxorubicin released from the niosomes was about 10 fold greater than the clearance of niosomal doxorubicin (176.5 mL h^{-1} and 16.2 mL h^{-1}, respectively). The area under the plasma level-time curve increased 6 fold when doxorubicin was administered in niosomes, compared to doxorubicin solution (66.0 μg.h mL^{-1} and 10.3 μg.h mL^{-1}, respectively). The area under the tumour level time curve was increased by over 50% by the administration of doxorubicin in niosomes when compared to the drug administered in solution (58.6 μg.h mL^{-1} and 34.3 μg.h mL^{-1}, respectively). There was no statistically significant difference between levels of the drug in the heart when niosomal doxorubicin or doxorubicin solution were administered. Doxorubicin metabolites, namely doxorubicinol and the aglycones doxorubicinone, doxorubicinolone and 7-deoxydoxorubicinone, were found associated with the niosomes in the plasma, possibly due to their adsorption to the vesicle surface once formed outside the niosome. Overall metabolite levels in the liver were increased when doxorubicin niosomes were administered compared to the drug in solution. A 5 mg kg^{-1} injection of doxorubicin niosomes produced a terminal mean tumour weight that was similar to that obtained from animals administered 10 mg kg^{-1} doxorubicin solution. ***Conclusions.*** Modest tumour targeting was achieved by the delivery of doxorubicin in sorbitan monostearate niosomes, increasing the tumour to heart AUC^{0-24} ratio from 0.27 to 0.36 and a doubling of tumoricidal activity. The overall level of doxorubicin metabolites was also increased.

KEY WORDS: doxorubicin; niosomes; tumoricidal activity; metabolism; cholesterylpolyoxyethylene ether; sorbitan monostearate.

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INTRODUCTION

The use of doxorubicin in the treatment of solid tumours is hampered by the onset of a dose limiting cardiomyopathy and myelosuppression (1), among other adverse effects. A number of drug delivery systems have been developed in an attempt to maximize the benefits of doxorubicin chemotherapy, most having some element of drug targeting as an objective. Polymeric particulate systems (2,3) and liposomes (4–8) have been employed in an effort to achieve drug localization within neoplastic tissue and thus reduce the incidence of systemic toxicity. The intravenous administration of doxorubicin polyisohexylcyanoacrylate nanoparticles increased blood and reticuloendothelial system levels of the drug and reduced levels in the heart (2). While cardiac levels (7) and cardiotoxicity (4) appear to be reduced on liposomal encapsulation, myelotoxicity remains a problem with these formulations (6,9).

In this paper we present data on doxorubicin administered in niosomes which are non-ionic surfactant vesicles formed in a similar manner to liposomes (10) and offering structural and compositional alternatives to liposomes (11). Niosomes prepared from alkyl ether surfactants have previously been evaluated as carriers of doxorubicin in certain murine tumour models, bearing either mouse carcinomas (12) or human xenografts (13,14). In the present work, we have employed a widely available non-ionic alkyl ester surfactant, sorbitan monostearate (Span 60), used in foodstuffs and pharmaceuticals, to formulate doxorubicin loaded niosomes and have studied doxorubicin disposition, metabolism and tumoricidal activity in a tumour bearing mouse model (15).

The metabolism of doxorubicin is complex (16) (Figure 1), involving principally carbonyl reduction at the C13 side chain, reductive glycosidic cleavage at the C7 position, hydrolytic glycosidic cleavage and various other minor pathways such as O-demethylation, O-sulfation and O-β-glucoronidation. Of these, carbonyl reduction by way of the aldo-ketoreductase enzymes is the major enzymatic conversion encountered in humans giving rise to doxorubicinol. Although a large number of studies report the altered pharmacokinetics of doxorubicin in vesicular delivery, demonstrated by increased levels of doxorubicin in the plasma compartment (7,8), few reports deal with the impact of encapsulation on metabolism. Earlier work has found increased reductive glycosidic cleavage of doxorubicin, giving rise to elevated liver levels for the 7-deoxyaglycone metabolites (12,13).

MATERIALS AND METHODS

Doxorubicin hydrochloride, doxorubicinol hydrochloride, doxorubicinolone, doxorubicinone, 7-deoxydoxorubicinone were gifts from Farmitalia Carlo Erba, Italy. Sorbitan monostearate was obtained from Fluka Chemika Ltd, Germany, Choleth-24 (a 24 unit oxyethylene cholesteryl ether) from D. F. Anstead, UK and both cholesterol and epirubicin from Sigma Chemical Co, UK. Female NMRI mice were bred by one of us (J.A.D.) in the Clinical Oncology Unit,

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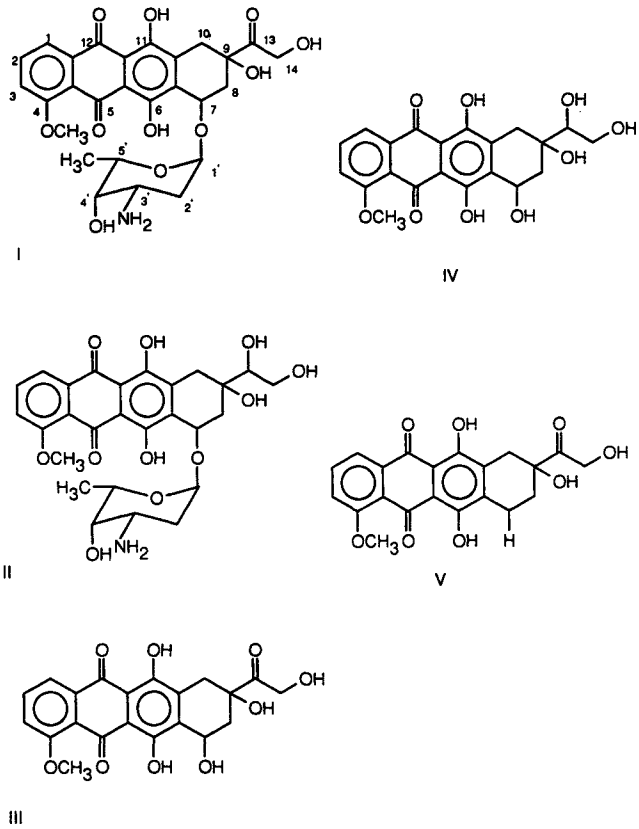

Fig. 1. Doxorubicin and its metabolites. I = Doxorubicin, II = doxorubicinol, III = Doxorubicinone, IV = Doxorubicinolone, V = 7-Deoxydoxorubicinone.

University of Bradford, UK and male BALB/c mice were purchased from Bantim and Kingman, UK.

Preparation of Sorbitan Monostearate Niosomes

Doxorubicin sorbitan monostearate niosomes were prepared from sorbitan monostearate, cholesterol, Choleth-24 (45:45:10) in the manner previously described (17). All suspensions were freshly prepared the day before administration and stored at 4°C in the intervening time period.

Assay of Doxorubicin in Niosomes

The sizing and assay of doxorubicin niosomes were performed using photon correlation spectroscopy and high performance liquid chromatography with fluorescence detection as described elsewhere (17).

Animal Investigations

Animals were housed in standard plastic cages at 19–23°C, with a 12 h light-dark cycle. A conventional diet (Rat and Mouse Standard, B&K Universal, Hull, UK) and water from the mains supply were available *ad libitum*.

Separation of Niosome Associated and Released Doxorubicin

Male BALB/c mice (n = 12) of mean weight 30.0 g were injected intravenously (IV) via the tail vein with either 5 mg kg^{-1} doxorubicin solution, or received no drug at all (controls). At 0 min (controls), 90 min and 4 h two mice were killed from each group by halothane overdose and blood collected by cardiac puncture, following a thoracotomy incision, into lithium heparin tubes. Plasma was immediately separated by centrifugation at 500 g. Fresh plasma, so obtained was fractionated over an 82 × 5 mm Sepharose 2B column eluted with TBS (pH 7.4), 25 0.27 ml fractions were collected, extracted and analyzed by HPLC. Both doxorubicin niosomes, doxorubicin solution and a mixture of doxorubicin niosomes and doxorubicin solution were also fractionated over 82 × 5 mm Sepharose 2B columns.

Pharmacokinetic Study on Tumour Bearing Animals

Female NMRI mice (mean weight 23.7 g) were implanted (day 0) subcutaneously with a MAC 15A tumour. On day 9, mice (n = 4) were administered intravenously with doxorubicin niosomes or doxorubicin solution (0.75 mg mL^{-1}) at a dose of 10 mg kg^{-1}. At the following time points 4 mice from each group were killed by halothane overdose: 0, 0.17 h, 0.5 h, 1.0 h, 2.0 h, 4.0 h, 8.0 h, 24 h. Blood was collected by cardiac puncture, following a thoracotomy incision, into lithium heparinized tubes. Plasma was separated from other blood components by centrifugation and divided into 2 portions. Half was stored at −44°C and the other half of the fresh plasma (50–100 μL) was fractionated over an 82 × 5 mm Sepharose 2B column. The column was eluted with TBS (pH 7.4). 2 fractions were collected; the void volume 1.8 mL (of which the first 0.5 mL were discarded) and a subsequent 3 mL fraction. Both samples were analyzed by HPLC. The liver, heart, tumour and lung tissue were also removed and immediately frozen in liquid nitrogen. Tissues were eventually stored at −44°C until analysis.

Tissue Analysis

HPLC analysis was a modification of the method of Cummings and associates (21). Tissue homogenates were extracted and analyzed by a modification of the method of Cummings and McArdle (19).

Assay for Plasma Proteins

50 μL plasma obtained from mice which had not been administered with doxorubicin (control mice) was passed over an 82 × 5 mm Sepharose 2B column eluted was TBS (pH 7.4). 25 0.27 mL fractions were collected and each assayed for total protein by the Lowry method.

Tumoricidal Activity of Doxorubicin Sorbitan Monostearate Niosomes

Male NMRI mice (n = 7, mean weight 23 g) bearing a subcutaneously implanted MAC 15A solid tumour were intravenously injected 6 days after tumour implantation with either 2.5, 5.0 or 10.0 mg kg^{-1} doxorubicin in sorbitan monostearate niosomes; 5 or 10 mg kg^{-1} doxorubicin solution; empty (encapsulating TBS) niosomes or TBS alone. The latter two groups served as controls. Tumoricidal indices were analyzed using one way analysis of variance (ANOVA).

The students t-test was used to compare doxorubicin tissue levels.

Pharmacokinetic Calculations

Non-compartmental methods were used for the calculation of all pharmacokinetic parameters (20). AUC^{0-24} was calculated by the linear trapezoidal rule, while $AUC^{24-\infty}$ was calculated by dividing the 24 h concentration time point by the terminal elimination rate constant (Cp_{24h}/β). Volume of distribution was calculated using the equation given below

$$Vd = \text{dose } AUMC/(AUC)^2 \quad (1)$$

AUMC = area under the first moment curve.

RESULTS AND DISCUSSION

Sorbitan monostearate niosomes into which doxorubicin was encapsulated with an efficiency of 35% had a mean diameter of 235 nm. The doxorubicin to lipid (surfactant + cholesterol) molar ratio was 0.015.

Separation of Niosome Associated and Released Doxorubicin in Plasma

In plasma samples fractionated by passage over Sepharose 2B (Figure 2), niosomes eluted in the void volume (elution volume 1.4 mL), while released doxorubicin eluted later (elution volume 3.4 mL). Samples from animals administered doxorubicin niosomes showed doxorubicin eluting in the void volume, while doxorubicin in samples from animals given doxorubicin solution co-eluted with plasma proteins (Figure 2). Free doxorubicin is largely plasma protein bound (21). Freshly prepared doxorubicin niosomes eluted in the void volume as a single band (elution volume 1.4 mL, % recovery 89.5%) and plain doxorubicin solution eluted as a single band (elution volume 3.4 mL, % recovery 87.7%).

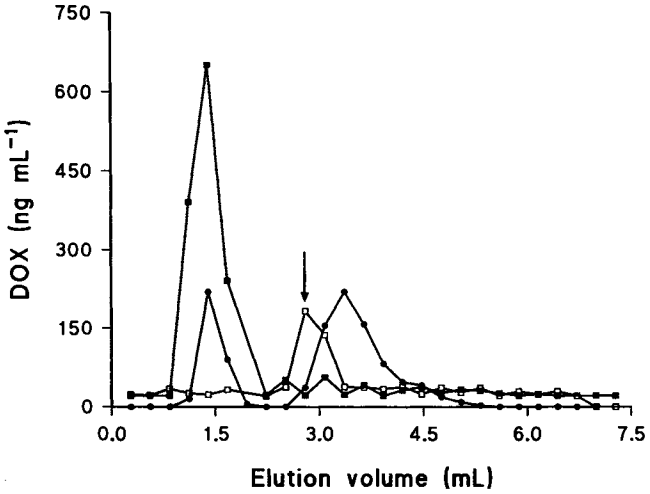

Fig. 2. Elution profile for various *in vivo* and *in vitro* samples fractionated over an 82 × 5 mm Sepharose 2B column. ■ = mouse plasma 90 min after intravenous injection (IV) of 5 mg kg^{-1} doxorubicin sorbitan monostearate niosomes. □ = mouse plasma 90 min after IV 20 mg kg^{-1} doxorubicin solution. ● = doxorubicin solution + doxorubicin sorbitan monostearate niosomes. Arrow = Peak of plasma protein elution. Plasma proteins elute as a coloured distinct band.

Pharmacokinetic Studies

The area under the plasma level time curve $AUC^{0.17-24}$ was increased 6 fold by niosomal encapsulation (Figure 3) from 10 μg.h mL^{-1} to 66.0 μg.h mL^{-1}. Four hours after dosing, 90% of the plasma doxorubicin was still associated with the niosomes and 50% was still associated 24 h after dosing (Figures 4 & 5), indicating hitherto unreported *in vivo* stability of the doxorubicin niosomes. Phospholipid vesicles, show varying levels of associated doxorubicin *in vivo*, depending on the rigidity of the liposome membrane. Phosphatidylcholine and phosphoglycerol vesicles show 50–90% of the drug to be vesicle associated up to 2 h after dosing (5) and for the more rigid hydrogenated phosphatidylcholine vesicles, 57% of plasma doxorubicin is vesicle associated 72 h after dosing (22). Other plasma pharmacokinetic parameters are given in Table I. The clearance and volume of distribution of doxorubicin was decreased quite substantially by niosomal encapsulation, evidence that doxorubicin niosomes are confined to a large extent to the vascular space. The released doxorubicin (Figure 4), shows typical biexponential clearance kinetics. In the terminal elimination phase, the curve actually rises, most likely due to the input from degraded doxorubicin niosomes.

Levels of doxorubicin in the liver were doubled with the niosomal formulation (Figure 6) and tumour levels were increased by 50% (Figure 6). Doxorubicin levels in the lung were also increased by niosomal encapsulation (AUC^{0-24} = 1632 μg.h mL^{-1} and 727 μg.h mL^{-1} for doxorubicin niosomes and doxorubicin solution respectively) while heart levels were unaffected (Figure 6) by the formulation used. The depot nature of doxorubicin sorbitan monostearate niosomes ensured an increased level of doxorubicin in the tumour. Tumour/heart AUC^{0-24} ratio was also increased from 0.27 with doxorubicin solution to 0.36 with doxorubicin niosomes. Similar improvements in the tumour/heart drug level

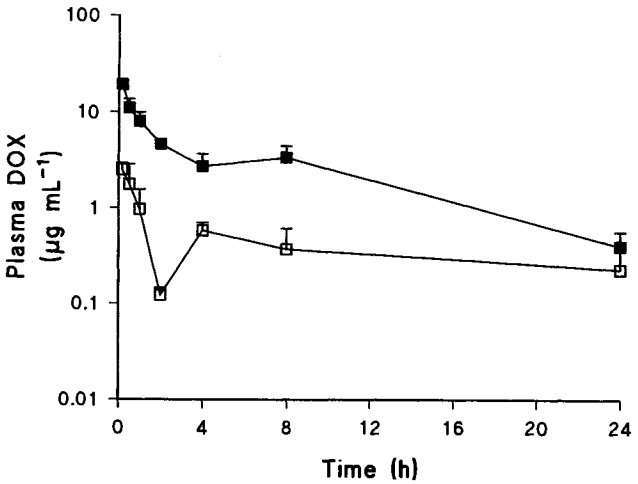

Fig. 3. Mean plasma levels (± s.e) of doxorubicin after IV injection of doxorubicin sorbitan monostearate niosomes (10 mg kg^{-1}) and doxorubicin solution (10 mg kg^{-1}) to female NMRI tumour bearing mice. ■ = doxorubicin sorbitan monostearate niosomes, □ = doxorubicin solution.

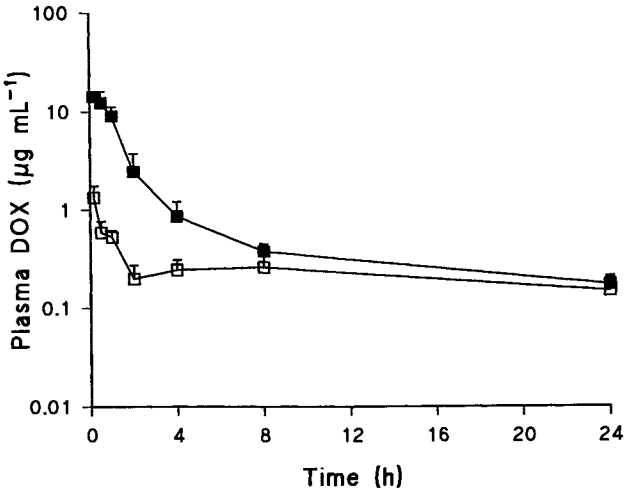

Fig. 4. Mean plasma levels (± s.e.) of doxorubicin after IV injection of doxorubicin sorbitan monostearate niosomes (10 mg kg^{-1}) to female NMRI tumour bearing mice. ■ = niosome-associated doxorubicin. □ = released doxorubicin.

ratio have been reported for other doxorubicin alkyl ether niosomes (11) and doxorubicin phospholipid vesicles (8). Doxorubicin niosomes were mainly taken up by the liver with 40% of the dose being found in the liver 10 min after dosing—including the accompanying organ blood pool.

Metabolites

The method of separation used unlike similar methods (23,24), allows direct quantification of released doxorubicin and its metabolites. Metabolites were detected and quantified in both the plasma niosomal and released drug fractions. The AUC^{0-24} values of the various metabolites were higher in the niosomal fraction than in the released drug fraction. This association of doxorubicin metabolites with niosomes could have arisen due to the adsorption of the metabolites, formed outside the vesicles to the vesicle surface. Liver metabolites from both formulations were also quantified (Table II) and with the niosomal formulations there were higher AUC^{0-24} values for all the metabolites quantified (Table II). Increased liver levels of 7-deoxydoxorubicinone were also reported on administration of doxorubicin alkyl ether niosomes (12,13). Despite the fact that the synthesis of 7-deoxydoxorubicinone is increased in the liver on administration of the vesicular formulation and its formation *in vitro* is accompanied by free radical generation (16), this reaction has been ruled out as a mechanism of doxorubicin cytotoxicity (25) and the synthesis of 7-deoxydoxorubicinone *in vivo* is considered to be a deactivating transformation.

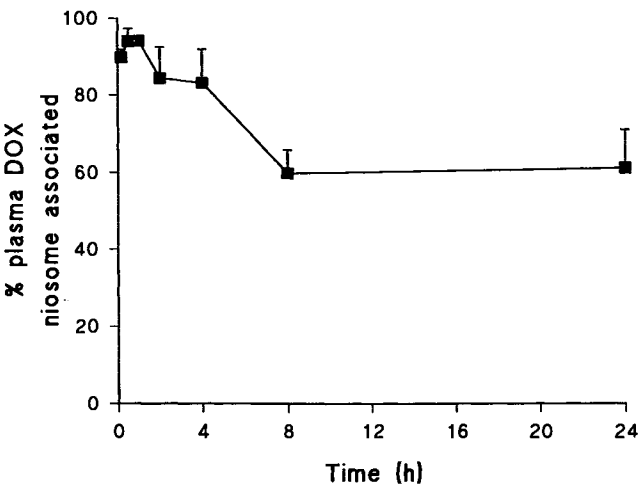

Fig. 5. Mean percentages of plasma doxorubicin (± s.e.) still encapsulated in niosomes after IV injection of doxorubicin sorbitan monostearate niosomes (10 mg kg^{-1}) to female NMRI tumour bearing mice.

Table I. Pharmacokinetic Parameters for Doxorubicin Solution and Doxorubicin Sorbitan Monostearate Niosomes

Doxorubicin formulation	Volume of distribution (mL)	Clearance (mL min^{-1})[a]
Total doxorubicin from niosomes (encapsulated + released)	13.6	4.2
Niosome associated doxorubicin	—	8.1
Released doxorubicin	—	20.7
Doxorubicin solution	66.4	18.2

[a] For a 30 g mouse.

The high levels of doxorubicin metabolites found in the liver on the administration of doxorubicin niosomes is in stark contrast to the reduced dose-standardized level of 7-deoxydoxorubicinone found between 10 and 40 min after dosing, on administration of elevated doses. This dose dependence is not observed over a 48 h period. This suggests

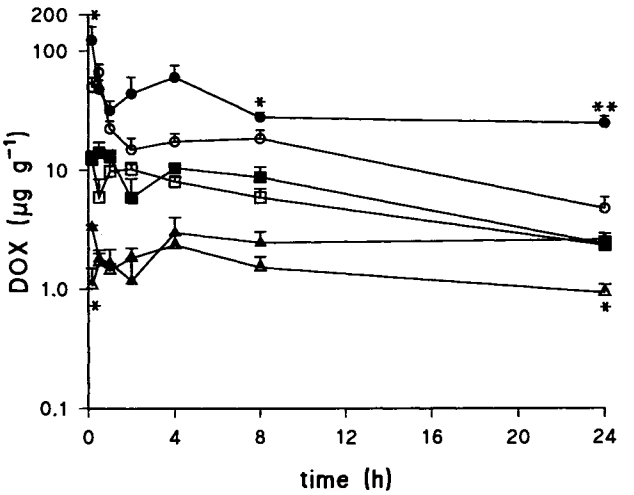

Fig. 6. Mean tissue levels (± s.e.) of doxorubicin after IV injection of doxorubicin sorbitan monostearate niosomes (10 mg kg^{-1}) and doxorubicin solution (10 mg kg^{-1}) to female NMRI tumour bearing mice. ● = liver levels after the administration of doxorubicin sorbitan monostearate niosomes, ○ = liver levels after the administration of doxorubicin solution, ■ = heart levels after the administration of doxorubicin sorbitan monostearate niosomes, □ = heart levels after the administration of doxorubicin solution, ▲ = tumour levels after the administration of doxorubicin sorbitan monostearate niosomes, △ = tumour levels after the administration of doxorubicin solution, * = statistical significance ($p < 0.05$), ** = statistical significance ($p < 0.01$).

Table II. Doxorubicin Metabolite AUCs and $AUC_{(metabolite)}$ $AUC_{(doxorubicin)}$ in the Plasma, Liver and Lung After the Administration of Doxorubicin Sorbitan Monostearate Niosomes and Doxorubicin Solution

Doxorubicin metabolite/tissue	Metabolite $AUC^{0.17-24}$ ($AUC_{(metabolite)}$) ($\mu g \cdot h\ mL^{-1}$)
Plasma niosome	
associated doxorubicinol	5.2
doxorubicinone	0.32
7-deoxydoxorubicinone	0.12
Plasma unencapsulated fraction after doxorubicin niosome administration	
doxorubicinol	1.26
doxorubicinone	0.23
7-deoxydoxorubicinone	0.085
Liver metabolites after doxorubicin sorbitan monostearate niosomes	
doxorubicinol	54.2
doxorubicinone	4.0
13-dihydrodoxorubicinone	0.53
7-deoxydoxorubicinone	0.75
Liver metabolites after doxorubicin solution	
doxorubicinol	12.9
doxorubicinone	2.7
13-dihydrodoxorubicinone	0.045
7-deoxydoxorubicinone	0.19

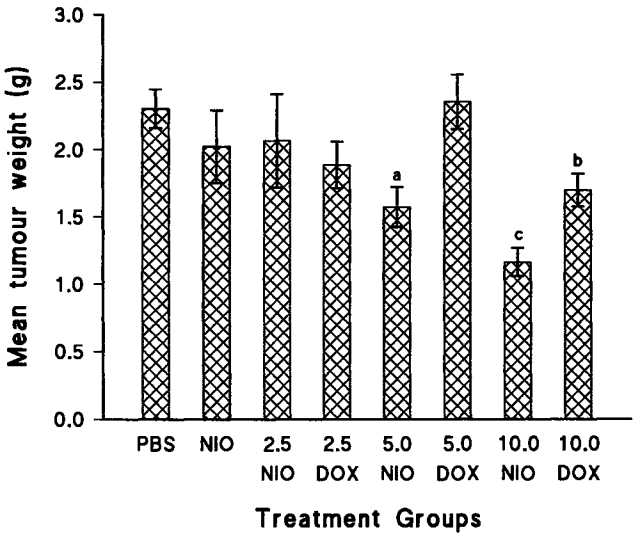

Fig. 7. The tumoricidal activity of intravenously injected DOX Span 60, cholesterol, Solulan C24 (45:45:10) niosomes against a MAC 15A tumour subcutaneously implanted in female NMRI mice. (expressed as mean tumour weight ± s.e.). PBS = PBS (pH 7.4), NIO = empty niosomes (encapsulating PBS – pH 7.4), 5.0 DOX and 10.0 DOX = 5.0 and 10 mg kg^{-1} doxorubicin solution, 2.5 NIO, 5.0 NIO, 10.0 NIO = 2.5, 5.0 and 10.0 mg kg^{-1} doxorubicin in niosomes. The differences between a and b are not statistically significant, while the differences between c and b were statistically significant.

that the rate of synthesis of this aglycone is reduced at high doses although the elimination kinetics are non capacity-limited (26). Also the intravenous administration of niosomal methotrexate to mice resulted in increased liver levels of the main metabolite 7-hydroxymethotrexate (27).

Tumoricidal Activity

Span 60 niosomes used against the MAC 15A tumour had a mean diameter of 171 nm and a doxorubicin/lipid molar ratio of 0.014. Empty Span 60 niosomes had a mean diameter of 132 nm. When these Span 60 niosomes were evaluated against an established (7 day old) MAC 15A tumour they were found to be twice as active as the drug in solution (Figure 7) ($p < 0.05$). 5 mg kg^{-1} doxorubicin Span 60 niosomes were equiactive with 10 mg kg^{-1} doxorubicin solution ($p > 0.05$). 10 mg kg^{-1} DOX Span 60 niosomes gave % inhibition values of 49.6%, compared to 26.5% given by 10 mg kg^{-1} doxorubicin solution. Results on the tumoricidal activity of Span surfactant niosomes are not widely found in the literature, although a study of sonicated vincristine loaded Span 60 niosomes did indicate an improvement in tumoricidal activity with multiple doses of Span 60 vincristine niosomes (28). The present work however, is the first demonstration of improved tumoricidal activity with doxorubicin Span 60 niosomes.

Model tumours have been used to assess the efficacy of liposomal doxorubicin formulations with varying results, determined not only by intrinsic tumour differences, but also by bilayer characteristics, dosing regimens (multiple dosing versus single doses) and administration routes. Large multilamellar phosphatidylcholine and phosphatidylserine vesicles evaluated against transplanted metastatic murine lymphomas (J6546) (7) and a metastatic murine mastocytoma (29) showed no improvement over the activity of doxorubicin administered as the free drug in solution, whether repeat (29) or single (7) dosing was employed. Work with single doses of intravenously injected sonicated (65–100 nm) doxorubicin hydrogenated phospholipid vesicles showed improved tumoricidal activity in an ascitic form of the murine lymphoma (J6456) (8), expressed as improved survival. Multiple intravenous injections of sonicated doxorubicin phosphatidylserine-phosphatidylcholine liposomes only marginally increased survival times with the metastatic murine lymphoma (J6456) (7). When multiple dosing and the use of a surface polyoxyethylene compound was employed (31) improved prophylaxis against spontaneous metastases (with MC19 and MC65 tumours) and increased tumoricidal activity against recently implanted tumours (MC2A, MC2B & MC65) was observed. Similar results with multiple dosing regimens were observed with the use of C-26 colon carcinomas in mice (31).

CONCLUSIONS

Doxorubicin niosomes were found to circulate *in vivo* acting as a depot within the plasma. This resulted in an improvement in the overall tumour to heart drug levels. The tumoricidal activity of the doxorubicin sorbitan monostearate formulation was also superior to the drug in solution. Doxorubicin metabolites were found associated with doxorubicin niosomes *in vivo*, probably due to the adsorption of these compounds to the outside of the bilayer or due to the degradation of the parent molecule within the bilayer. The

level of doxorubicin liver metabolites were increased by niosomal encapsulation although the exact impact of the quantitative metabolism differences between free and niosomal doxorubicin remains to be assessed. Data presented herein supports the conclusion that there are advantages in the delivery of doxorubicin in niosomes which require pursuit.

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